PATENT

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Allan C. **SPRADLING** *et al.*

Application No.: 09/358,937 — Group Art Unit: 1632

Filed: July 23, 1999 — Examiner: J. Woitach

For: **METHOD FOR MAINTENANCE AND PROPAGATION OF GERMLINE STEM CELLS USING MEMBERS OF THE TGF-β FAMILY OF GROWTH FACTORS**

Commissioner for Patents
Washington, D.C. 20231

Sir:

considered
/jw 3/3/03

DECLARATION UNDER 37 C.F.R 1.132

I, Allan C. Spradling, declare as follows:

1. I am a co-inventor of the subject matter described and claimed in the above-referenced patent application, U.S. Patent Application No. 09/358,937 (the '937 application).

2. I have read and understand the non-final Office Action (paper no. 20) that was mailed July 17, 2002, in the '937 patent application, particularly the rejections under 35 U.S.C. 102(b) based on Twombley *et al.* (hereinafter Twombley) and Forbes *et al.* (hereinafter Forbes). It is my understanding that a basis for both rejections is that, even though the references do not

specifically report that an abundance of germline stem cells was achieved, the method of providing dpp to germline cells using a transgene as taught by the references would inherently lead to an increased abundance of stem cells as claimed. It is my understanding that a further basis for the Forbes rejection is that the ectopic expression of dpp and the increased number of germline cells observed by Forbes demonstrate that Forbes teaches the limitations of the claimed invention. I will address each of these rejections individually, beginning with the rejection based on the Forbes reference.

3. By 1996, when the Forbes paper was published, my laboratory was the world's leading authority on Drosophila germline stem cells. We verified that these stem cells existed in 1993 by laser ablation (Lin *et al.*, 1993). We discovered the molecular composition of the stem cell cytoskeleton known as the fusome and reported on antibodies that allow stem cells to be readily identified by immunostaining in 1994. In 1995 we published studies of these cells using sophisticated lineage tracing methods that revealed many new insights into their behavior and also revealed the existence, number and location of the somatic stem cells (Margolis and Spradling, 1995). Thus, by the time the Forbes reference was published, my laboratory had developed technology for determining the number, location and type of stem cells present in the Drosophila ovary.

4. The studies reported by Forbes *et al.* were initiated and mostly carried out in my laboratory, and was under my personal supervision and participation. They were done in the hopes of finding that hedgehog, wg and/or dpp were regulators of either germline stem cells, somatic stem cells or both. At the time the studies were performed, we had the interest, experience and methodology to determine if either cell population responded to the genetic manipulations under study. No significant increases in the number of germline stem cells were observed, despite the fact we analyzed the relevant tissues using sensitive methods and were

hoping for just such changes. Consequently, I can attest to the fact that no significant increase in the number of stem cells inherently occurred in these experiments.

5. The Office Action asserts that the increased number of cells observed by Forbes demonstrates that Forbes teaches the limitations of the claimed invention. However, what my colleagues and I observed, as reported in the Forbes paper, was the formation of abnormal follicles containing a multiplicity of germline cysts. Germline cysts are sets of 16 differentiated germ cells, not stem cells. Furthermore, the multiplicity of cysts comes at the expense of the total number of follicles (normally there should be 7 follicles, not 3 as shown). Thus, the total number of differentiated germ cells observed in the Forbes paper was actually the same number as seen in wild type. They were just packaged into half the number of follicles. We now know that the effects observed and reported in this paper were achieved because dpp has a separate role 4 days after its role in stem cells- in the process of follicle formation. Follicle formation has nothing to do with germline stem cells. Cystoblasts, and all of the other differentiated types of germ cells, are distinct from germline stem cells, and can be distinguished from germline stem cells in the proteins they express (for instance, cystoblasts express cytoplasmic Bam protein whereas germline stem cells do not; their fusomes also differ). We carried out relevant antibody stainings and identified the state of differentiation of the germ cells reported in this paper. Thus, in the Forbes paper, we reported changes in the behavior of differentiated germ cells, not an abundance of germline stem cells as claimed. When we did look at the status of stem cells in these hs-dpp flies we saw no significant increases. At that time, there was no reason to think that the lack of an increase in the number of stem cells was unusual, and hence there was no reason to report this observation in the Forbes paper.

6. Turning now to the Twombley reference, although the work reported in Twombley was carried out in another laboratory, the authors sent me examples of their data as well as early drafts of their paper prior to submission in hopes that I (as a friend and ovary expert) might

provide additional insight into the effects they were observing. First, of all, most of Twombley *et al.*'s experiments reduced dpp signaling, and would not have been expected to cause an increase in the number of stem cells. Only the hs-dpp strain (which we tested using the techniques described above and found it not to work) and the 8X dpp strain might have been expected to have such an effect if they were activated properly. Later, when Dr .Xie joined my lab, we obtained the 8X dpp strain and studied it in my lab. Neither Twombley *et al.*, nor I in the data they provided, observed any substantial increase in stem cells.

7. There are several very simple reasons why we can be confident that no significant increase in the abundance of stem cells took place in the experiments reported in Twombley. The events studied by Twombley *et al.* occur late in oogenesis, about six days after the stem cell stage, and just a day or two before eggs are laid. The Twombley paper starts by incorrectly determining that dpp is only expressed late in oogenesis (just as we failed to see early expression in Forbes *et al.*). Consequently, they focused only on these late stages. As a result, they did not follow an appropriate protocol of heat shocks to get an effect on stem cells. For instance, as reported in the specification (page 27, line 5), multiple heat shocks over 3-5 days would be needed at a minimum to observe a significant effect on the number of stem cells. However, because they were looking at late follicles, where effects are seen in just 1-2 days, only very short periods of heat shock of a few days were used. Thus, only negligible effects would even have been expected in their experiments. Moreover, if stem cell number had been significantly increased, late developmental stages would have been rare or entirely absent. That is because the extra stem cells come from a blockade of development. Germ cells become extra stem cells instead of follicles. In our experience, the production of new follicles is completely blocked before significant numbers of stem cells accumulate. Thus, the fact that Twombley *et al.* continued to observe and study follicles in their experiments tells us that there was not a block of germ cell development sufficient to significantly increase the number of stem cells.

8. Even if Twombley *et al.* had carried out what we only later discovered was an appropriate protocol of heat shocks, they might not have seen a stem cell increase. That is because the heat shock promoter-based constructs they used do not appear inherently to be very good reagents for this purpose. Both the heat shock-dpp transgene used by Forbes *et al.* or the 4 transgene insertions used to increase the number of copies of the "wild type" dpp gene in the 8X dpp strain by Twombley *et al.* are structurally abnormal, and lack some of the control sequences that drive gene expression *in vivo*. The endogenous dpp gene is large and complicated; it contains at least 4 alternate promoters as well as 40 kb or more of control sequences located to the 5', 3' and possibly within introns of these transcripts. The hs-dpp gene lacks all these control sequences and relies on the heat shock promoter to express Dpp protein. The construct used in the 8X dpp strain, is basically the same as the hs-dpp construct with the addition of some of the 3' control sequences. However, it probably lacks the source of the control sequences normally driving dpp expression early in oogenesis. This control element behaves in an unusual manner because the dpp-lacZ strain 10638 (which was originally constructed and studied in my lab) and which Twombley *et al.* partly relied on in concluding that dpp was not expressed early in oogenesis, mimics the expression of the normal dpp gene in all other tissues, in late oogenesis, but not in early oogenesis.

9. The second critical factor that often strongly affects the performance of constructs, is their chromosomal insertion site(s). Each site has particular, unpredictable "position effects" on the expression of the construct. The sites at which the hs-dpp constructs were inserted in the strains used by Twombley *et al.* and Forbes *et al.* may have blocked or reduced expression in the relevant cells surrounding the germ line stem cells. Germ cells and surrounding somatic cells are particularly resistant to manipulation of gene expression, possibly reflecting mechanisms that protect them from parasitism by exogenous viruses, transposons, etc. For instance, for reasons that are not understood, heat shock promoters cannot be activated in germ cells during most of oogenesis.

10. Even when a promoter does work, there are corresponding problems that may arise at every subsequent level of gene expression: splicing, transport, translation, protein folding, protein modification, etc. that may prevent the final delivery of an adequate level of a functional protein product. The physical structure of the transcripts produced by transgenes are not identical to wild type. Consequently, the cellular machinery for processing and translating those transcripts does not always function in the expected manner, and these reactions differ in an unpredictable way from cell type to cell type.

11. We eventually got different dpp constructs to work (for instance, hs-GAL4, UAS-dpp constructs) as described in our disclosure and paper (Xie and Spradling, July 24, 1998, Cell 94: 251-60) (of record). The best results were obtained by mating UAS-dpp lines with particular GAL4 driver lines as disclosed in the specification (see, for instance, page 27, lines 1-5). Even then, we had to screen hundreds of driver lines, but eventually found suitable lines that could increase stem cell number by a thousand fold or more.

12. In conclusion, as a co-author of the Forbes paper and a contributor of the work reported therein, I can attest that no significant increase in the abundance of germline stem cells was achieved in that study. Further, as an expert in the field of Drosophila germline stem cells, I am confidant that no such increase in the abundance of stem cells was achieved by Twombley *et al.* in view of the late application of a rather short heat shock regimen and the observation of later follicular developmental stages that do not occur when stem cells divide and increase substantially in number rather than differentiate. There are several other factors that may have contributed to the lack of increase in stem cell number in these studies, including the nature of the constructs used and the integration sites of the constructs. Nevertheless, while the invention is based on the discovery that dpp can be provided to germline stem cells so as to increase their abundance, finding an appropriate expression construct/driver line, etc., was part of the general

state of the art of a skilled worker in the field at the time. It is important to understand that without knowledge of the invention in hand, a random attempt to express dpp in Drosophila is unlikely to work, and in fact did not work historically.

13. I further declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true, and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

By: _____

Dated: 1/15/03